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Porcine Sialoadhesin: A Newly Identified Xenogeneic Innate Immune Receptor

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Abstract

Extracorporeal porcine liver perfusion is being developed as a bridge to liver allotransplantation for patients with fulminant hepatic failure. This strategy is limited by porcine Kupffer cell destruction of human erythrocytes, mediated by lectin binding of a sialic acid motif in the absence of antibody and complement. Sialoadhesin, a macrophage restricted lectin that binds sialic acid, was originally described as a sheep erythrocyte binding receptor. Given similarities between sialoadhesin and the unidentified macrophage lectin in our model, we hypothesized porcine sialoadhesin contributed to recognition of human erythrocytes. Two additional types of macrophages were identified to bind human erythrocytes - spleen and alveolar. Expression of sialoadhesin was confirmed by immunofluorescence in porcine tissues and by flow cytometry on primary macrophages. A stable transgenic cell line expressing porcine sialoadhesin (pSn CHO) bound human erythrocytes, while a sialoadhesin mutant cell line did not. Porcine macrophage and pSn CHO recognition of human erythrocytes was inhibited approximately 90% by an anti-porcine sialoadhesin monoclonal antibody and by human erythrocyte glycoproteins. Furthermore, this binding was substantially reduced by sialidase treatment of erythrocytes. These data support the hypothesis that porcine sialoadhesin is a xenogeneic receptor that mediates porcine macrophage binding of human erythrocytes in a sialic acid-dependent manner.

Keywords

Macrophages; liver; lectin; sialic acid; xenotransplantation

INTRODUCTION

Within the last decade, innate immune cells, specifically macrophages, have gained attention in the field of xenotransplantation due to their contribution to a new form of xenograft rejection not seen in allotransplantation—a process known as delayed xenograft rejection (1). Delayed xenograft rejection, or acute vascular rejection, is not completely understood, but it is composed of both innate and adaptive immune responses in the organ recipient (2). The innate cellular immune response found in porcine hearts rejected by baboons consists of

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injuries inflicted by baboon natural killer cells and macrophages (3). The best evidence for baboon macrophage destruction of porcine cells comes from studies infusing porcine peripheral blood progenitor cells into baboons (4). In these studies, depletion of baboon recipient macrophages with medronate liposomes resulted in delayed clearance of porcine cells, suggesting that baboon macrophages bind and eliminate porcine cells by either antibody-dependent cell-mediated cytotoxicity or direct recognition by specific macrophage receptors. Human Kupffer cells, macrophages of the liver, directly recognize porcine erythrocytes without the need for opsonization, suggesting that primate macrophages bind porcine cells using innate immune cell surface receptors (5). Thus, it appears that abrogation of macrophage recognition and destruction of xenogeneic cells will be an important accomplishment in the goal of achieving long-term xenograft survival.

Our laboratory studies how macrophages recognize non-self—specifically how porcine macrophages recognize human erythrocytes. This focus developed from efforts to design a treatment for patients in fulminant liver failure using extracorporeal porcine liver perfusion (6). This treatment option could provide for patients in fulminant liver failure a bridge to liver transplantation or allow recovery without a liver transplantation. During a 72-hour porcine liver perfusion with human blood, a decrease in hematocrit is observed when perfusing livers from both wild-type and human decay accelerating factor transgenic pigs (7), resulting from porcine Kupffer cells binding human erythrocytes (8). In extracorporeal porcine liver perfusion experiments lasting 72 hours, porcine Kupffer cells bind and destroy more than 90% of the perfused human erythrocytes (7). This observation was the opposite of what was expected. Traditionally in solid organ allotransplantation and xenotransplantation, the main risk is that the host destroys the graft; but in this model, the graft was attacking host cells. This mechanism of destruction does not appear to be mediated through complement or antibody mediated opsonization (9), but rather, involves direct recognition by a macrophage receptor (8). Further studies demonstrated that porcine Kupffer cells bind a human erythrocyte carbohydrate ligand (10); specifically, these cells recognize a sialic acid motif on human glycophorin A (11). We hypothesized that macrophages have carbohydrate-specific lectin receptors that mediate direct recognition of unopsonized xenogeneic cells (12).

Sialoadhesin, or siglec-1, is a macrophage restricted lectin that binds sialic acid (13, 14). Historically, sialoadhesin was identified as a bone marrow macrophage receptor that mediated recognition of sheep erythrocytes in attempts to understand the presence of resident bone marrow macrophages and their cellular functions (14). Although characterization of the exact biological function of sialoadhesin remains uncertain, more general roles for sialoadhesin have been identified in the areas of cancer biology (15, 16), erythropoiesis(17), inflammatory conditions (18, 19) and clearance of pathogens (20–22). N*eisseria meningitides* is one of the few pathogens where sialoadhesin has been shown to mediate macrophage recognition of non-self (20). Sialoadhesin has been shown to mediate internalization (23) and endocytosis (24) of PRRSV, porcine reproductive and respiratory syndrome virus, into macrophages. In an interesting twist, PRRSV directs its host cell to glycosylate viral surface glycoproteins so that PRRSV is bound by sialoadhesin expressed on porcine alveolar macrophages; the virus thereby targets these cells for infection (21). It is worth noting that the ability of sialoadhesin to mediate macrophage recognition of non-self was the defining characteristic for which this molecule was originally named – the sheep erythrocyte binding receptor (25). This cellular interaction is a form of xenogeneic recognition – mouse macrophage recognition of sheep erythrocytes. While most researchers have viewed this interaction as an oddity limited to the laboratory, we propose that this xenogeneic interaction contributes to the understanding of how macrophages recognize xenogeneic epitopes in the field of xenotransplantation.

Given the work identifying porcine sialoadhesin as a sialic acid-binding porcine macrophage receptor involved in PRRSV infection, we hypothesized that this same receptor might be responsible for mediating porcine Kupffer cell recognition of human erythrocytes. We provide evidence that sialoadhesin mediates porcine macrophage recognition of human erythrocytes and that inhibitors of this process block both porcine macrophage binding of human erythrocytes and PRRSV infection of porcine alveolar macrophages.

MATERIALS AND METHODS

All animal experiments were approved by the University of Toledo IACUC. Large white pigs were obtained from a local pig farm (15–20 kg) and treated in accordance with the ILAR and the Animal Welfare Act (26). Blood was collected from either piglets or blood group O human volunteers. Written informed consent was obtained for all human volunteers under a University of Toledo IRB approved protocol.

Virus

The European prototype PRRSV strain Lelystad virus (kindly provided by G. Wensvoort) and the Belgian PRRSV strain, 94V350 (27) were used in these experiments (28). Details regarding passaging and infection rates in porcine alveolar macrophages are previously described (27).

Cells

After macrophage isolation, preparations were incubated overnight to select for adherent cells. Flasks were then washed with the appropriate medium and returned to the incubator for one week before utilization. Generally, macrophage cultures were viable for 2–3 weeks. Unless specified, all mediums and supplements were obtained from Life Technologies (Carlsbad, CA).

Kupffer cells and erythrocytes—Porcine Kupffer cells, human erythrocytes, and porcine erythrocytes were isolated as previously described (8).

Spleen macrophages—Splenectomy was followed by hepatectomy. Residual blood was removed by perfusion of the splenic artery with ice cold saline (Baxter, Deerfield, IL). The spleen was minced and processed with 1 L of cold PBS (Oxoid Inc., Ogdensburg, NY) through 500, 212, and 106 micron metal sieves (CSC Scientific Inc., Fairfax, VA). This cellular solution was equally distributed into six 250 mL bottles and brought up to a final volume of 200 mL with PBS + 10% FBS. The resulting cellular solution was incubated on ice for 30 min. The supernatant was then centrifuged at 600 × g for 5 min. The resulting pellets were combined and brought up to a final volume of 225 mL with PBS + 10% FBS. This cellular solution was layered over Ficoll-Paque PLUS, (GE Healthcare Life Sciences, Piscataway, NJ) and centrifuged for 45 min at 3007 × g. The interface was carefully removed and washed in HBSS + 10% FBS, and centrifuged at 469 × g for 7 min. Finally, the pellet was washed with medium and placed in culture. Spleen macrophages were maintained in RPMI (Cell Gro, Herndon, VA), 1% penicillin/streptomycin (100 U/mL, 100 μg/mL), 10% FBS, and 2.7% mM L-glutamine, 200 mM.

Alveolar macrophages—Porcine alveolar macrophages were collected by performing a broncho-alveolar lavage on adult pigs and frozen for long-term use as previously described (29). Thawed alveolar macrophages were used approximately 4 days after being in culture.

pSn CHO, pSn^{RE} CHO, and wild type CHO cells—CHO-K1 cells (ATCC, CCL-61) were maintained in F12 Medium supplemented with 5% FBS, 1% sodium pyruvate, and 1%

penicillin/streptomycin (100 U/mL, 100 μg/mL). The construction of the full-length sialoadhesin mutant, which lacks sialic acid binding activity (pSn^{RE}), and the generation of stable CHO cell line that expresses porcine sialoadhesin (pSn CHO) are described previously (30).

Chromium^{51} Rosetting Assay

Binding—Quantification of porcine macrophage recognition of human erythrocytes has been described previously (8). Briefly, potential inhibitors were applied to macrophages prior to the addition of ^{51}Chromium labeled erythrocytes. Unbound erythrocytes were washed away and gamma radiation measured. This binding assay was adapted to measure binding between CHO cell lines and human erythrocytes. CHO cells were applied to glass cover slips and incubated at 37°C for 48 hrs. Prior to the application of potential inhibitors, all CHO cell samples were treated with *Vibrio cholerae* sialidase (Roche, Indianapolis, IN). Sialidase removes cell surface sialic acid that contributes to cis-inhibition of sialoadhesin. 8 mU of sialidase, diluted in RPMI medium, was applied to each cover slip for 30 min and washed twice.

Inhibition of binding with human erythrocyte glycoproteins (hEGP)—Both hEGP and porcine erythrocyte glycoproteins (pEGP) were prepared previously (10).

Inhibition of binding with pSn mAb—The murine monoclonal antibody 41D3 against porcine sialoadhesin (pSn mAb) was described previously (31, 32). A purified mouse IgG monoclonal antibody served as the isotype control (BD Biosciences, Franklin Lakes, NJ).

Sialidase treatment of human erythrocytes—Human erythrocytes were treated with sialidase (Roche, Indianapolis, IN). 1.0 μL of packed erythrocytes was re-suspended in 400 μl of RPMI medium and treated with 20 mU of sialidase to remove terminal sialic acid. Erythrocytes were incubated with sialidase for 1 hr at 37°C. Erythrocytes were washed once to remove released sialic acid prior to ^{51}Chromium labeling.

Flow Cytometry

All treatments were done for 30 min on ice. Since macrophages contain Fc receptors, cells were blocked with 20 μL of neat horse serum per sample. Primary antibodies included pSn mAb and the isotype control mouse anti-T-2 mycotoxin monoclonal antibody (Southern Biotech, Birmingham, AL). A goat anti-mouse IgG Alexa Flour 647 (Life Technologies, Carlsbad, CA) secondary antibody was used. Samples were washed twice between treatments with PBS. Samples were analyzed on a Cytometrics FC 500 flow cytometer (Beckman Coulter, Brea, CA). Data was analyzed using FlowJo, version 7.6.4

Quantification of PRRSV Infection of Alveolar Macrophages

Macrophages were fixed and stained as previously described (28). Briefly, infected cells were detected by immunoperoxidase staining with a monoclonal antibody (P3/27) against the PRRSV nucleocapsid protein (33) followed by incubation with horseradish peroxidase-labeled goat anti-mouse secondary antibody and then developed with 3-amino-9-ethylcarbazole as a substrate.

Immunohistochemistry

Porcine macrophages were incubated with human and porcine erythrocytes as described for the ^{51}Chromium rosetting assay, except that the erythrocytes were not radioactively labeled. Macrophages were fixed in a 1:1 acetone/methanol solution. The primary antibody mouse anti-pig monocyte/granulocyte (BD Biosciences, Franklin Lakes, NJ) was used to identify

porcine macrophages. Porcine erythrocytes were identified with the primary antibody mouse anti-pig CD235a monoclonal antibody, (BD Biosciences, Franklin Lakes, NJ) and human erythrocytes were identified with a mouse anti-human glycophorin A monoclonal antibody (DakoCytomation, Carpinteria, CA). Images were taken with an Olympus BHS (Center Valley, PA) microscope equipped with an Evolution MP 5.0 Mega-pixel Color Real Time Viewing digital camera (Media Cybernetics, Silver Spring, MD) and analyzed with Q Capture Pro 5.0 software (Mager Scientific, Dexter, MI).

Confocal Microscopy

Frozen sections were cut with a cryostat and prepared on positively charged glass microscope slides. All incubations were done in a humidified box at 37°C. pSn mAb was labeled with FITC (Zenon Alexa Fluro 488 labeling kit) according to the manufacturer's instructions (Life Technologies). A comparable mouse IgG_1 isotype control was labeled to serve as a negative control. Slides were analyzed on a TCS SP5 multiphoton laser scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL).

Statistical Analysis

Data collected for the quantitative ^{51}Chromium rosetting assay was analyzed using descriptive statistics. Each sample for each experiment was performed in triplicate and the standard error for each sample calculated from the standard deviation of the three values obtained. Each experiment was completed a minimum of three times. The average values of each experiment were then used to calculate the standard error for all three experiments.

RESULTS

Porcine spleen and alveolar macrophages bind human erythrocytes

While porcine Kupffer cells have previously been demonstrated to bind human erythrocytes (8), we evaluated the ability of porcine spleen and alveolar macrophages to bind human erythrocytes using both a quantitative ^{51}Chromium rosetting assay (Figure 1A) and a qualitative immunohistochemistry assay (Figure 1B). Porcine spleen and alveolar macrophages bound human erythrocytes, but not porcine erythrocytes (Figure 1 A and B). These data demonstrate that at least three resident porcine tissue macrophages are capable of binding human erythrocytes. Given the difficultly and expense of procuring, and the limited yield of macrophages obtained from the liver and spleen, alveolar macrophages were utilized as a substitute for Kupffer cells given their ease of procurement and large yields (~10^7 Kupffer cells obtained per pig liver vs. 3×10^9 alveolar macrophages per broncho-alveolar lavage).

Porcine sialoadhesin is expressed in porcine liver and lung tissues, in vivo and in vitro

We hypothesized that porcine sialoadhesin is a macrophage receptor that mediates porcine macrophage recognition of human erythrocytes. Thus, we wanted to confirm that porcine sialoadhesin is expressed in porcine liver and lung tissues and in primary macrophages. In Figure 2A, flow cytometry analysis of primary macrophages demonstrated surface expression of sialoadhesin on porcine Kupffer cells (MFI of 2,700.3 ± 609.6) and alveolar macrophages (MFI of 3,586.3 ± 572.6) as compared to the isotype control (478.5 MFI ± 27.1). In Figure 2B, qualitative immunofluorescence of liver and lung tissues demonstrated varied levels of sialoadhesin expression at low and high levels of magnification. It's not surprising that we found sialoadhesin expression levels vary among resident macrophages as this has been demonstrated with other species of macrophages (14, 25, 34, 35). In regards to how levels of sialoadhesin expression contribute to porcine Kupffer cell recognition of human erythrocytes, about 20 erythrocytes are bound per macrophage; therefore, levels of

sialoadhesin expression are not indicative of binding. More likely though, binding is limited by steric hindrance. These data confirm that porcine sialoadhesin is naturally expressed *in vivo* and that expression *in vitro* is not an artifact of isolation.

PRRSV infection of porcine alveolar macrophages and porcine alveolar macrophage recognition of human erythrocytes share two common inhibitors: human erythrocyte glycoproteins (hEGP) and an anti-porcine sialoadhesin monoclonal antibody (pSn mAb)

Given our previous observation that porcine Kupffer cells bind human erythrocytes via the terminal sialic acid on human glycophorin A (10, 11), and knowing that the sialic acid-binding lectin responsible for PRRSV entry into porcine alveolar macrophages is sialoadhesin (32), we examined whether two known inhibitors of each model (hEGP and pSn mAb) would reciprocally inhibit the other model in hopes of identifying the porcine macrophage receptor that mediates recognition of human erythrocytes. As demonstrated in Figure 3A, hEGP inhibited infection of porcine macrophages by PRRSV in a concentration dependent manner. Complete inhibition of infection was observed at a concentration of 500 μg/mL. To verify this same observation with porcine alveolar macrophages, alveolar macrophages were incubated with a reciprocal dilution of hEGP. As with porcine Kupffer cells (10), hEGP inhibited porcine alveolar macrophage recognition of human erythrocytes in a concentration dependent manner resulting in approximately 90% inhibition at a concentration of 500 μg/mL (Figure 3B). Porcine erythrocyte glycoproteins were used as a negative control at the highest concentration tested and binding was not inhibited. The pSn mAb at a 1:10 dilution inhibited porcine alveolar macrophage binding of human erythrocytes by nearly 95% (Figure 3C) and porcine Kupffer cell recognition of human erythrocytes by nearly 90% (Figure 3D). These data suggest that sialoadhesin is the porcine macrophage lectin responsible for the binding of human erythrocytes and further support sialoadhesin as the viral receptor for PRRSV.

Porcine sialoadhesin-expressing stable cell line (pSn CHO) binds human erythrocytes and binding is inhibited by both pSn mAb and hEGP

To examine the role of porcine sialoadhesin in porcine macrophage recognition of human erythrocytes, pSn CHO and a stable cell line expressing a mutant form of sialoadhesin (pSn^{RE} CHO; containing a sialic acid-binding domain that is disrupted) were tested for their ability to bind human erythrocytes. Prior to performing rosetting assays, sialoadhesin expression was confirmed on pSn CHO and pSn^{RE} CHO cell lines. In addition, absence of porcine sialoadhesin expression was confirmed in wild type CHO cells by flow cytometry, data not shown. The pSn CHO bound human erythrocytes (represented as 100% binding), but only 11.8% of porcine erythrocytes were bound (Figure 4A). Neither the negative control cell line (pSn^{RE}) nor wild type CHO-K1 cells bound human or porcine erythrocytes, < 12% (Figure 4A) which would be expected since CHO-K1 cells are epithelial cells and sialoadhesin is a macrophage-restricted lectin.

To further test the hypothesis that porcine sialoadhesin mediates porcine macrophage recognition of human erythrocytes, the inhibitors that blocked binding in the *in vitro* macrophage experiments were repeated with the pSn stable cell line. Both pSn mAb and hEGP inhibited pSn CHO recognition of human erythrocytes (Figures 4B and 4C). In Figure 4B, the pSn mAb inhibited binding in a serial dilution manner such that at a 1:10 dilution, binding was inhibited by approximately 95% compared to isotype control treated samples. Likewise, hEGP inhibited erythrocyte binding in a concentration dependent manner with 97.6% inhibition at a concentration of 500 μg/mL compared to 7.2% inhibition with pEGP at the same concentration.

pSn CHO and porcine alveolar macrophages do not bind sialidase treated human erythrocytes

Previously, we demonstrated the importance of sialic acid in porcine Kupffer cell recognition of human erythrocytes by showing that sialidase treatment of human erythrocytes eliminates binding (10). To verify this same observation with porcine alveolar macrophages and pSn CHO, human erythrocytes were sialidase treated and binding was measured using the ^{51}Chromium rosetting assay. Similar to porcine Kupffer cells, sialidase treatment eliminated the binding of human erythrocytes by both porcine alveolar macrophages and pSn (Figure 5). Binding of sialidase treated erythrocytes by alveolar macrophages was reduced by 92.2% and 98.4% for pSn CHO.

DISCUSSION

These data suggest that porcine macrophage binding of human erythrocytes is mediated by sialoadhesin. *In vitro* experiments demonstrated that porcine macrophage recognition of human erythrocytes is inhibited by both human erythrocyte glycoproteins (hEGP) and a monoclonal antibody directed against porcine sialoadhesin (pSn mAb). A porcine sialoadhesin stable cell line (pSn CHO) bound human erythrocytes in contrast to the mutant cell line pSn^{RE}; this binding to pSn CHO was inhibited by both pSn mAb and hEGP. In addition, these inhibitors not only block the xenogeneic model of porcine macrophage recognition of human erythrocytes, but also viral infection of porcine macrophages by PRRSV.

The critical role of carbohydrate recognition in xenotransplantation was first recognized with the discovery of the importance of the carbohydrate epitope galactose α1,3 galactose or Galα1→3Gal (36–38). The Galα1→3Gal epitope is present on all porcine endothelial cells (38, 39), but absent on human and other Old World primate cells (40). Hyperacute rejection has largely been overcome by our understanding of the role of preformed natural antibodies directed against Galα1→3Gal and the production of Galα1→3Gal deficient pigs (41). Humans differ from pigs not only in lacking the Galα1→3Gal epitope, but also in lacking the most common form of sialic acid expressed in all other mammals. Humans have lost the ability to express N-glycolylneuraminic acid due to a deletion mutation in the gene of the enzyme CMP-N-acetylneuraminic acid hydroxylase which converts N-acetylneuraminic acid to N-glycolylneuraminic acid (42). Thus, whereas all mammals other than humans (including chimpanzees) express more N-glycolylneuraminic acid than N-acetylneuraminic acid, humans only express N-acetylneuraminic, leading to humans recognizing N-glycolylneuraminic acid as a foreign antigen. Some have suggested that this difference in sialic acid usage between humans and pigs accounts for the non-Galα1→3Gal epitope recognized by non-Galα1→3Gal antibodies contributing to xenograft rejection (43). What we have shown is the corollary—that pigs have innate cellular immune receptors capable of recognizing the difference in N-acetylneuraminic acid expression in pigs versus humans.

While the destruction of human erythrocytes during extracorporeal porcine liver xenoperfusion was the impetus for elucidating the underlying molecular mechanism, the identification of a lectin-carbohydrate recognition event in innate cellular xenogeneic recognition mechanisms has broader implications (6, 7, 12). For example, these graft vs. host recognition mechanisms will also play a role in bridging liver xenografts (44) and in lung xenotransplantation (45). While we studied a graft versus host response when viewed from the perspective of extracorporeal porcine liver perfusion as a treatment of fulminant hepatic failure, we study this model being cognizant that the knowledge obtained may one day provide insight into *host versus graft* delayed xenograft rejection. We propose that our observation plays an important role in understanding how host macrophages recognize xenogeneic tissue. Supporting this idea are many studies showing that in the absence of

macrophages, xenograft survival is increased (4, 46–56). Elucidating the mechanism(s) by which macrophages or other innate immune cells contribute to xenograft rejection, will enable the development of strategies to overcome this barrier.

For example, human monocytes have been shown to bind porcine endothelium via the interaction of galectin-3 and the xenoantigen Gal-α-(1,3)Gal-β(1,4)GlcNAc-R (57). Kwiatkowski and Itescu demonstrated that human monocytes use unidentified receptors to bind carbohydrates on xenogeneic porcine endothelial cells (58) capable of directly recognizing both a terminally sialylated porcine carbohydrate epitope (59) and the commonly expressed Galα1→3Gal epitope found on porcine endothelium (60). In addition, human natural killer cells have been shown to bind the Galα1→3Gal epitope on porcine endothelial cells in the absence of antibody and complement (61–63). In other species, rat Kupffer cells have been shown to bind human erythrocytes through the GalNAc/Gal-particle receptor in the absence of antibody and complement (64).

Given the role that we have established in this manuscript for sialoadhesin (siglec-1) and the established role for siglecs to bind sialic acid motifs, it is possible that other siglecs or other innate immune receptors contribute to xenogeneic recognition leading to delayed xenograft rejection. To date, sixteen human siglec proteins have been identified. Some bind N-acetylneuraminic sialic acid while others have specificities to N-glycolylneuraminic acid sialosides (65). The specificity we have identified in this study has highlighted one of the unique genetic differences between humans and chimpanzees (and all other animals), so that humans express only the precursor form of sialic acid that is used by all other mammals (42). This study shows how the porcine innate immune system capitalized on this difference to allow the recognition of human erythrocytes and illustrates a limitation of using non-human primates studies to model human extracorporeal porcine liver perfusion (66).

Even so, while pigs express primarily N-glycolylneuraminic acid, the precursor N-acetylneuraminic acid is also present, for example on the surface of porcine aortic endothelial cells (67) and in the porcine liver (68). Therefore, it is not clear why porcine macrophages do not bind self N-acetylneuraminic acid on the surface of porcine cells, yet recognize this structure on human erythrocytes. We provide three possible explanations for this observation. First, porcine sialoadhesin may recognize the different density or spacing of N-acetylneuraminic acid on human versus porcine erythrocytes. Second, porcine sialoadhesin may not bind self-sialic acid due to regulation of sialoadhesin binding through *cis* interactions with the macrophage glycocalyx (69). Third, porcine macrophages may not bind “self” because a negative signal is communicating, “do not eat me” signals. Sialoadhesin may deliver a positive signal that tells macrophages to bind and eliminate xenogeneic cells, or more likely pathogens such as PRRSV, while other receptor-ligand interactions such as CD47-SIRPα provide a negative signal communicating “do not phagocytose.”

It has been demonstrated in mice that erythrocytes lacking CD47 are readily cleared by splenic macrophages (70, 71). CD47 serves as an erythrocyte marker of self since erythrocytes lack MHC Class I expression. The CD47-SIRPα interaction has been studied in the field of xenotransplantation and molecular incompatibilities have been demonstrated between porcine CD47 and human SIRPα resulting in the phagocytosis of porcine erythrocytes by human macrophages (72). If the CD47/SIRPα interaction in the reverse combination – porcine SIRPα interaction with human CD47 – was comparable, then porcine sialoadhesin would recognize and bind xenogeneic cells because the macrophage would not receive a negative signal inactivating phagocytosis due to species-specific incompatibilities.

A porcine graft versus host response against humans is not limited to erythrocytes. Porcine enriched liver sinusoidal endothelial cells have been shown to mediate phagocytosis of human platelets by the asialoglycoprotein receptor-1 (73). Interestingly, fetuin (a glycoprotein composed of sialic acid-terminated β-glucans) did not prevent human platelet uptake by porcine endothelial cells, a distinction that might be due to the differences in glycosylation between platelets and erythrocytes or receptor expression on endothelial cells versus macrophages. While it is clear that lectins play an important role in innate cellular immunity against xenografts, recent data demonstrating that porcine Kupffer cells bind human platelets using CD18 emphasize that innate cellular recognition of xenografts is not limited to lectin-carbohydrate interactions (74).

We have observed a graft versus host innate cellular immune response to xenogeneic targets during extracorporeal porcine liver perfusion. Some may argue that Kupffer cell binding of human erythrocytes could be overcome by simply removing porcine macrophages. However, deletion of the macrophage progenitor cells has never been successfully accomplished, suggesting that it is an embryonic lethal knock-out. Alternatively, the macrophages could be removed prior to xenotransplantation by treating the organ with clodronate liposomes that when ingested by macrophages, release clodronate into the cytoplasm and induce apoptosis, killing the macrophage. While this approach may prevent loss of erythrocytes during extracorporeal liver perfusion, such pre-treatment may compromise the immune and physiological function of the liver, which may be one of the important benefits of extracorporeal liver perfusion. Another possible solution during extracorporeal liver perfusion would be to block porcine macrophage recognition of human erythrocytes using an inhibitor of sialoadhesin. Solid organ xenografting of liver or lung would require a permanent solution to sialoadhesin-mediated binding of human erythrocytes. Possibly, sialoadhesin knock-out pigs would provide the best solution for extracorporeal liver perfusion. Mice deficient in sialoadhesin have already been prepared and are viable and healthy (75).

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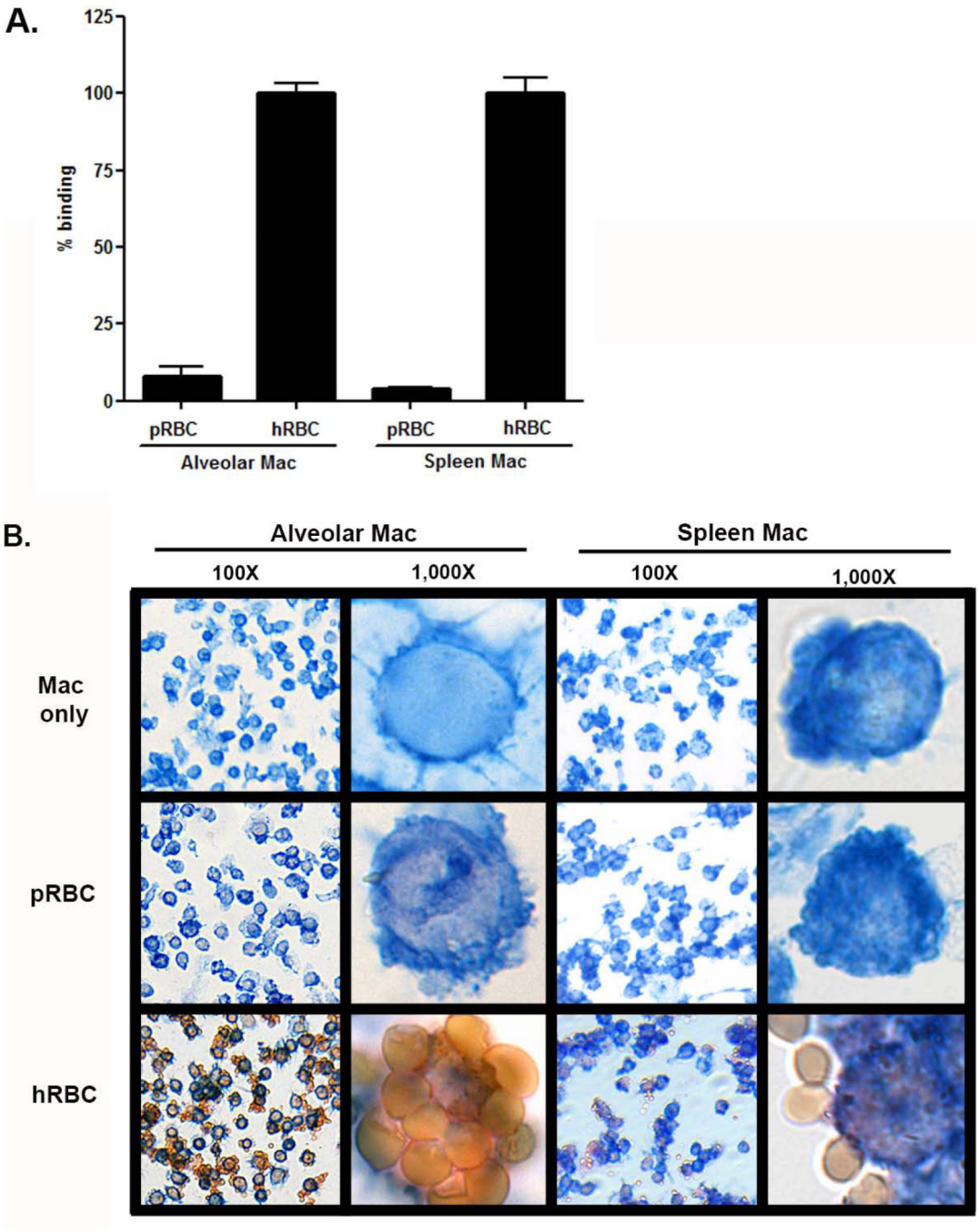

Figure 1. Porcine alveolar and spleen macrophages bind human erythrocytes and not porcine erythrocytes

Spleen macrophages (spleen mac) and alveolar macrophages (alveolar mac) were incubated with porcine erythrocytes (pRBC) and human erythrocytes (hRBC). **A.** Porcine macrophage binding of human erythrocytes was quantified using a ^{51}Chromium rosetting assay. Samples were prepared in triplicate and repeated 3 times, N=9. The means are shown. The standard error is calculated; however, for some samples it is very small and obscured by the bar. **B.** Macrophage recognition of human erythrocytes was visualized using two-color immunohistochemistry at 100X and 1,000X magnification. Porcine macrophages were identified with a primary antibody, anti-porcine macrophage antibody 74-22-15A, followed

by a biotinylated secondary antibody, and developed blue with a Vector Blue alkaline phosphatase substrate. Erythrocytes were identified using anti-porcine or anti-human glycophorin A followed by a biotinylated secondary antibody, and developed red with Vector Red alkaline phosphatase substrate. Samples were prepared in triplicate. Data is representative of two independent experiments.

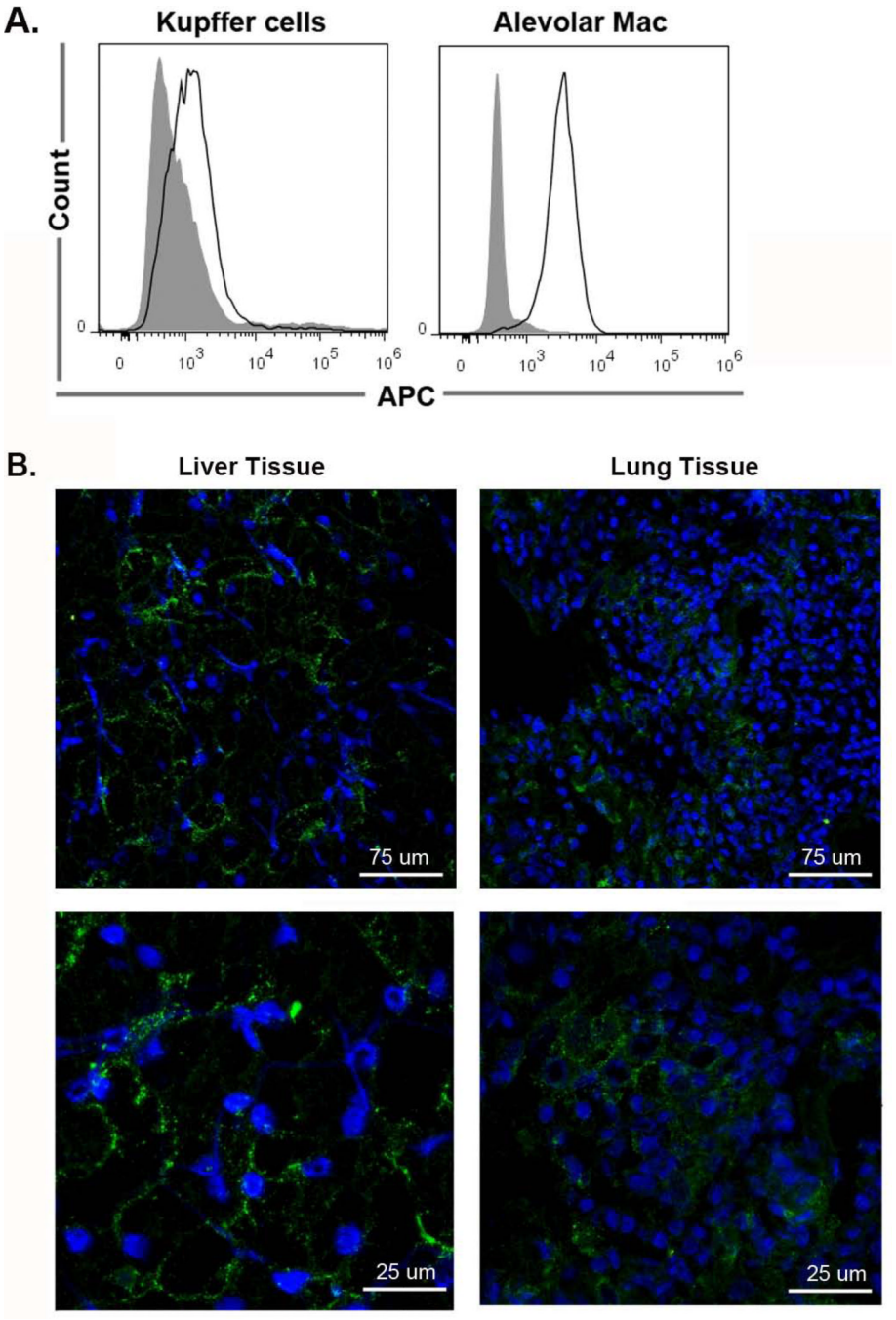

Figure 2. Confirmation that porcine sialoadhesin is expressed *in vitro* in primary cultures of Kupffer cells and alveolar macrophages and *in vivo* in liver and lung tissues

A. Porcine Kupffer cells and alveolar macrophages were evaluated by flow cytometry for expression of sialoadhesin. Macrophages were stained with an appropriate isotype control (filled, gray) and stained with the pSn mAb (outlined, black). 5×10^5 cells were used per sample. Data is plotted FSC (y axis) verses APC (x axis). This experiment was repeated three times. **B.** 1 cm^2 blocks of porcine liver and lung tissues were evaluated for expression of porcine sialoadhesin. Porcine tissues were placed in Tissue-Tek O.C.T. and prepared for cryosectioning. Porcine sialoadhesin was detected with Alexa 488 (green) and the nucleus was stained with DAPI (blue). Isotype control samples were negative (not shown). Images

were taken on a multiphoton laser scanning confocal microscope and representative z-stacks are shown. The upper panel images were taken at 40X and the lower panel images were taken at 40X zoomed in at a factor of 3.8. Samples were prepared in duplicate. Data is representative of two independent experiments. White measurement bars are included in each image for estimation of size.

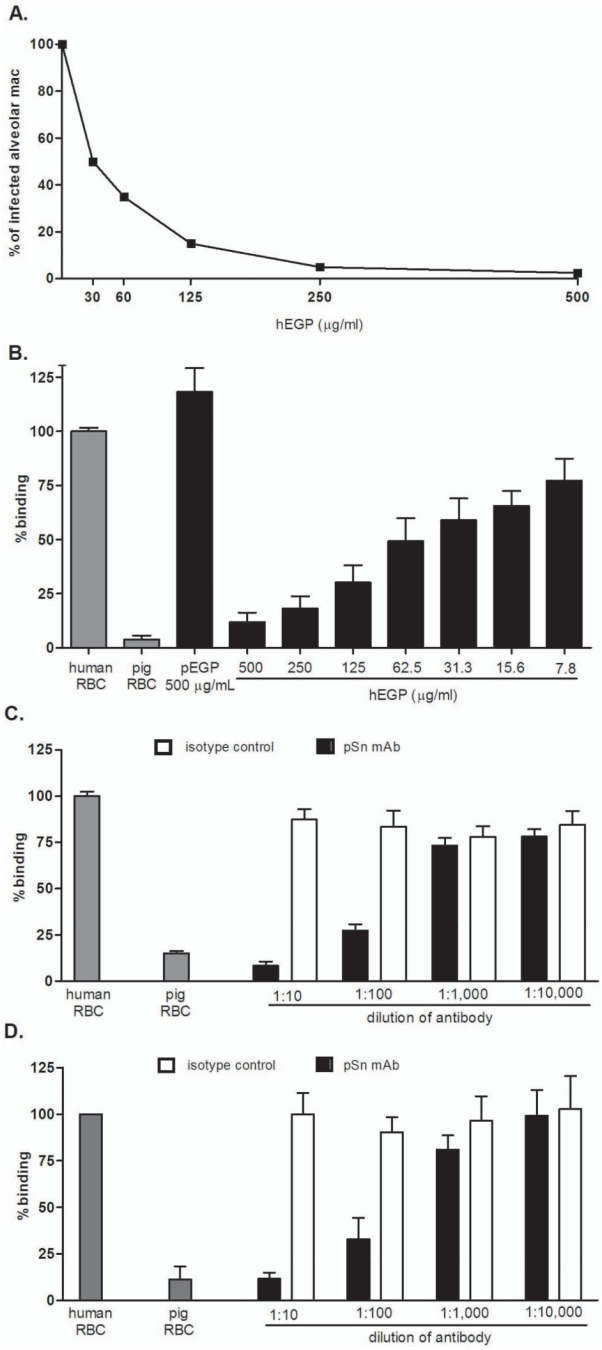

Figure 3. PRRSV infection of porcine alveolar macrophages and porcine alveolar macrophage recognition of human erythrocytes share two common inhibitors: human erythrocyte glycoproteins (hEGP) and an anti-porcine sialoadhesin monoclonal antibody (pSn mAb)

A. At a range of 500 to 0 μg/ml, hEGP was tested as a potential inhibitor of PRRSV infection of porcine alveolar macrophages. Inhibition was measured using immunofluorescence. Viral antigen-positive cells and total cells were counted with an Olympus light microscope and the percentage of infected cells calculated. Three microscope fields and a minimum of 100 cells per field were counted for each experimental condition. **B–D.** Potential inhibitors were evaluated using the ^{51}Chromium rosetting assay. Samples were prepared in triplicate. Data is representative of three independent experiments.. The

means are shown. The standard error is calculated; however, for some samples it is very small and obscured by the bar. Human and porcine erythrocytes in the absence of a potential inhibitor served as positive and negative controls (gray bars). **B.** hEGP was tested as a potential inhibitor of alveolar macrophage recognition of human erythrocytes at a range from 500 to 7.8 μg/ml. hEGP was prepared in RPMI medium. The negative control used in this experiment was pEGP, porcine erythrocyte glycoproteins. **C.** Inhibition of porcine alveolar macrophage recognition of human erythrocytes by pSn mAb was tested. A serial dilution of the pSn mAb ranging from 1:10 – 1:10,000 was prepared in RPMI medium and incubated with porcine alveolar macrophages. Mouse IgG was used as an isotype control. **D.** Inhibition of Kupffer cell recognition of human erythrocytes by pSn mAb was tested. pSn mAb was incubated with porcine Kupffer cells at a range of dilutions from 1:10 to 1:10,000. Mouse IgG was used as an isotype control.

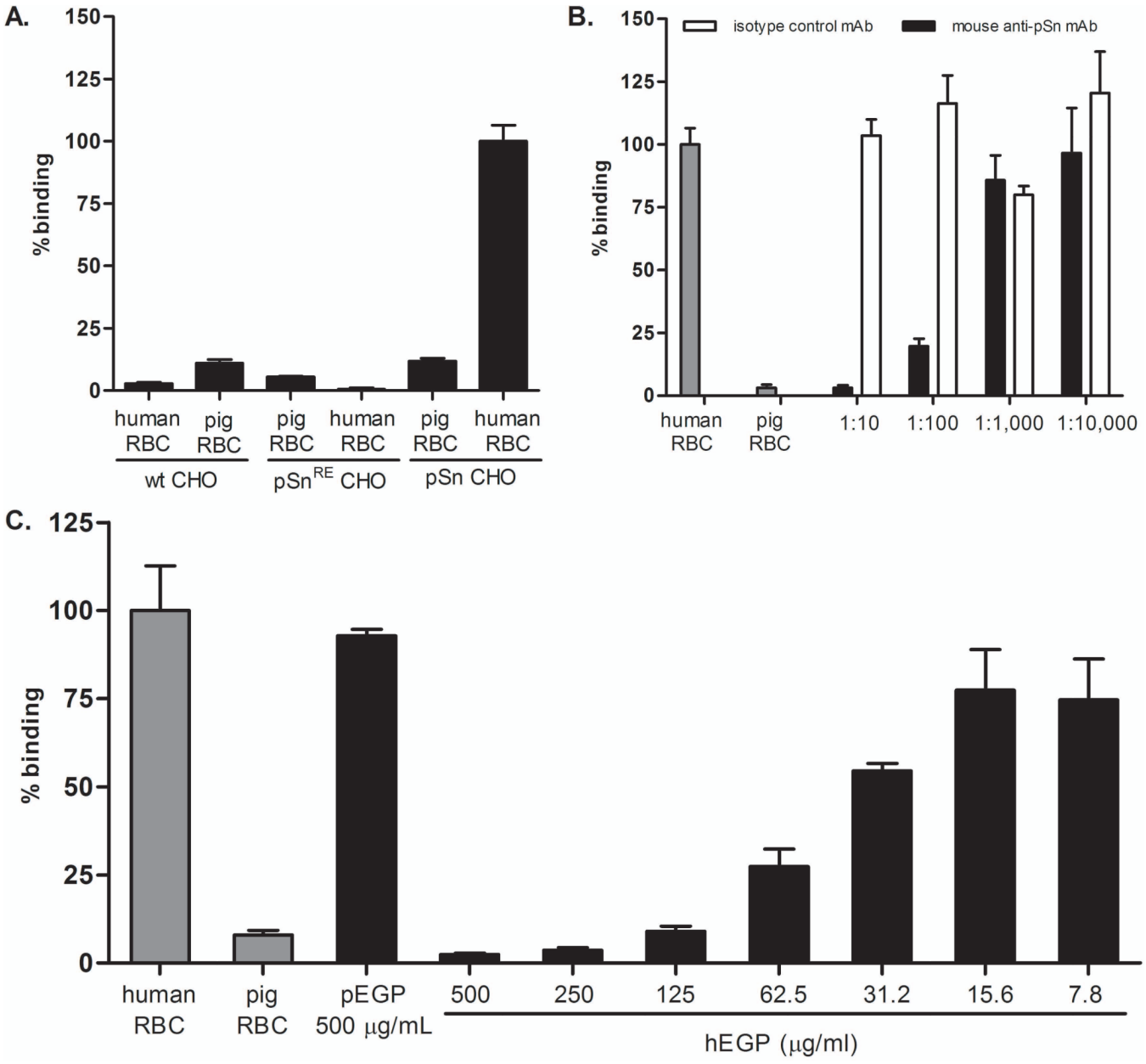

Figure 4. The sialoadhesin stable cell line (pSn CHO) binds human erythrocytes and this binding is inhibited by both the monoclonal antibody against porcine sialoadhesin (pSn mAb) and human erythrocyte glycoproteins (hEGP)

The pSn CHO cell was evaluated for its ability to bind human erythrocytes. For a negative control, a stable cell line expressing a mutant form of sialoadhesin (pSn^{RE} CHO) was used. Briefly, a sialic acid-binding mutant of sialoadhesin was generated by modifying the amino acid arginine 116 (which is by analogy to mouse sialoadhesin critical for the sialic acid-binding activity of porcine sialoadhesin) to a lysine residue by site directed mutagenesis (32). Binding in these experiments was quantified using the ^{51}Chromimum binding assay. Samples were prepared in triplicate. Data is representative of three independent experiments.. The means are shown. The standard error is calculated; however, for some samples it is very small and obscured by the bar. **A.** pSn CHO cells were evaluated for their ability to bind human erythrocytes. Wild type CHO cells and pSn^{RE} CHO served as negative

controls. **B.** A pSn mAb was tested as a potential inhibitor of binding. A comparable isotype control was used to show that inhibition of binding wasn't an artifact of steric hindrance. **C.** hEGP were tested as a potential inhibitor of binding at a range of 500 ug/mL to 7.8 ug/mL. Porcine erythrocyte glycoproteins (pEGP) were used as a negative control.

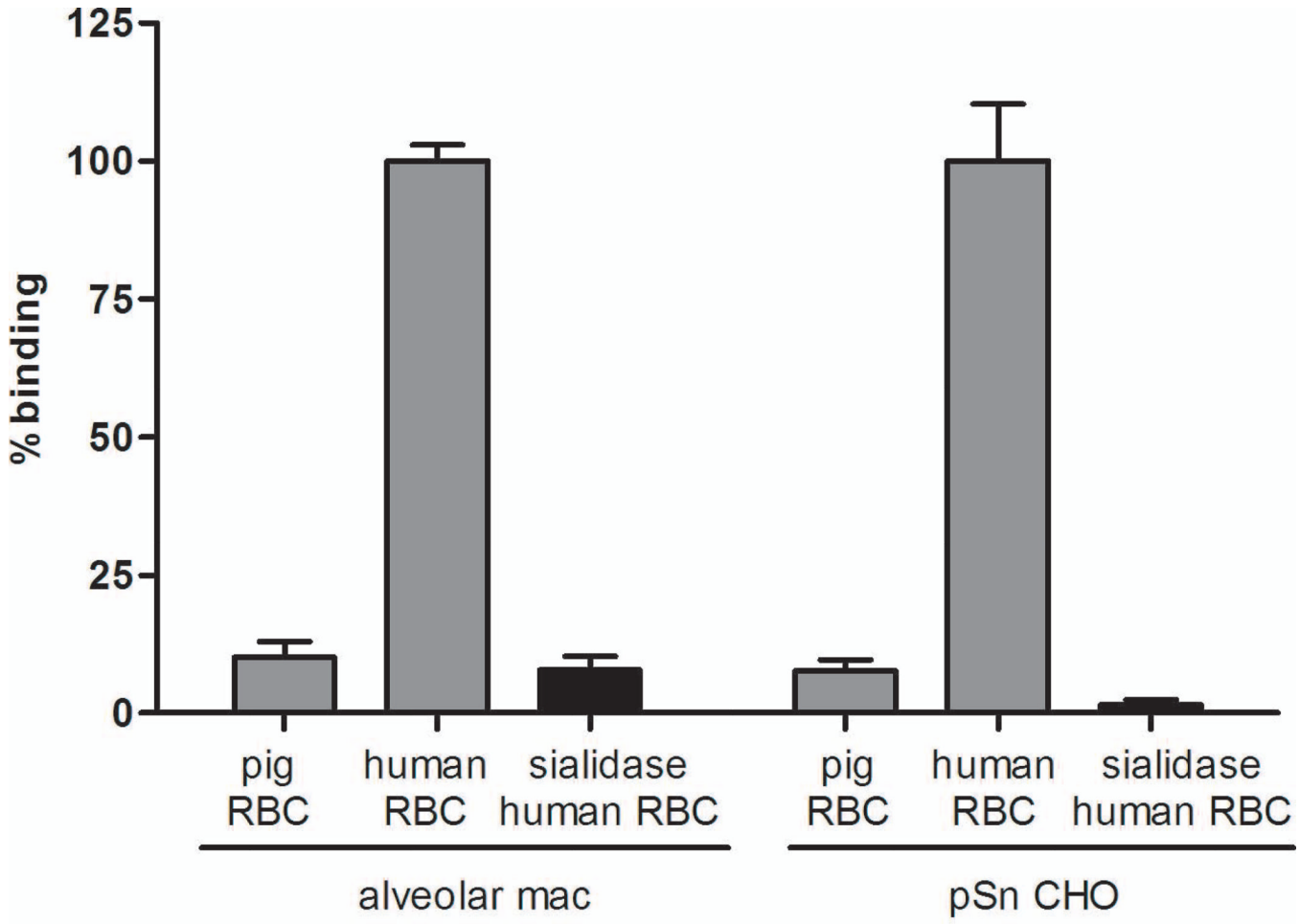

Figure 5. Sialic acid on human erythrocytes is necessary for the sialoadhesin stable cell line (pSn CHO) to recognize human erythrocytes

Human erythrocytes were treated with sialidase to remove terminal sialic acid and were then labeled with ^{51}Chromium. Untreated human and porcine erythrocytes were used as negative and positive controls. Erythrocytes were incubated with either alveolar macrophages (alveolar mac) or the porcine sialoadhesin stable cell line (pSn CHO). Binding in these experiments was quantified using the ^{51}Chromium Binding Assay. Samples were prepared in triplicate. Data is representative of three independent experiments.. The means are shown. The standard error is calculated; however, for some samples it is very small and obscured by the bar.